Transferrin-binding ability of invasive and commensal isolates of *Haemophilus* spp.

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Summary. *Haemophilus influenzae* type b expresses an inducible siderophore-independent iron-acquisition system that depends on a direct interaction between human transferrin and specific iron-regulated transferrin-binding outer-membrane proteins. To evaluate the importance of this iron-acquisition system amongst haemophili, 156 isolates of *Haemophilus* spp. (78 commensal isolates and 78 isolates from invasive infections) were examined for their ability to bind transferrin. Of the 78 invasive isolates, all of which were *H. influenzae* type b, 71 (91 %) were capable of binding transferrin, with 57 (73 %) binding transferrin constitutively (i.e., even when grown in an iron-sufficient medium). In contrast, only 11 (14 %) of the commensal isolates bound transferrin constitutively, with a further 16 (21 %) binding transferrin only after growth in an iron-deficient medium. Of the 27 commensal strains that were capable of binding transferrin, 12 were *H. parainfluenzae* biotype III, 14 were non-typable *H. influenzae*, and one was *H. parahaemolyticus*. None of the *H. influenzae* type b invasive or commensal isolates showed evidence of siderophore production, but 50 (66 %) of the remaining 76 commensal isolates appeared to produce an iron chelator. Thus, while not a universal characteristic, detectable transferrin-binding was associated strongly with *H. influenzae* type b isolates from invasive infections, and was also recognised for the first time in isolates of *H. parainfluenzae* and *H. parahaemolyticus*.

Introduction

Members of the genus *Haemophilus* are common commensals of the human upper respiratory tract,¹ but they are also capable of causing a variety of infections in man, ranging from serious invasive disease to minor opportunist infections. Thus, while *H. influenzae* type b is the most important cause of meningitis, epiglottitis, pneumonia and otitis media in young children,² other species such as *H. parainfluenzae*, *H. parahaemolyticus*, *H. aphrophilus*, *H. paraphrophilus* and *H. segnis* have been implicated, albeit infrequently, in a wide range of infections that includes pneumonia, septic arthritis, endocarditis, respiratory tract infection, bacteraemia and soft tissue infection.³

To cause infection, an organism must first acquire sufficient essential nutrients to multiply *in vivo*. One such essential nutrient is iron, and the low concentration of free iron on the mucous membranes and in tissue fluids is one of the first lines of host defence against bacterial infection.⁴ Pathogenic bacteria have developed several mechanisms for circumventing this defence mechanism and acquiring the necessary iron

for growth from their host. The best studied of these mechanisms involves iron chelators, termed siderophores, that compete with the host iron-binding glycoproteins lactoferrin and transferrin for iron, and iron-repressible outer-membrane proteins that serve as receptors for iron-siderophore complexes. This type of high-affinity iron-transport system has been detected in a wide range of bacterial pathogens,⁵ including *H. parainfluenzae* and *H. paraphrophilus*.⁶ Other pathogens, notably *Neisseria meningitidis* and *H. influenzae* type b, have been shown to express siderophore-independent iron-sequestering systems based on a direct interaction between host iron-binding glycoproteins, such as transferrin, and bacterial surface receptors (transferrin-binding proteins; TBPs).^{7–11}

In laboratory-adapted isolates of *H. influenzae* type b, such as strain Eagan,¹² expression of TBPs occurs only when iron is absent from the growth medium.¹¹ In contrast, examination of a small number of fresh clinical isolates of *H. influenzae* type b from the blood and cerebrospinal fluid (CSF) of patients with meningitis has shown that TBPs are expressed constitutively, i.e., even in the presence of excess free iron.¹³ Therefore, it appears that the selective pressure of growth in the iron-restricted environment of blood or CSF may result in a regulatory switch leading to

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constitutive expression of the TBPs. The aim of the present study was to evaluate the overall importance of the TBP iron-acquisition system amongst haemophili by determining the transferrin-binding ability of human commensal and invasive isolates belonging to a range of different *Haemophilus* species and biotypes.

Materials and methods

Bacteria

Invasive clinical isolates of *Haemophilus* spp. were isolated on chocolate blood agar¹⁴ in the routine diagnostic microbiology laboratory at University Hospital, Nottingham, from the blood cultures or CSF of patients suffering from meningitis (one strain/patient). Commensal strains of *Haemophilus* spp. were

obtained from randomly selected routine throat swabs (one strain/patient) cultured initially on a selective medium comprising chocolate blood agar containing vancomycin 5 mg/L. After overnight incubation at 37°C in CO₂ 5% in air, putative colonies of *Haemophilus* spp. were subcultured to purity on chocolate blood agar. The laboratory-adapted strain of *H. influenzae* type b, strain Eagan,¹² was kindly provided by Professor R. Moxon (Department of Paediatrics, John Radcliffe Hospital, Oxford). All haemophili were grown routinely on chocolate blood agar or in Brain Heart Infusion Broth (BHI; Oxoid CM225) supplemented with protoporphyrin IX (PPIX; Sigma) 0.5 mg/L and nicotinamide adenine dinucleotide (NAD; Sigma) 2 mg/L (sBHI). Strains of haemophili were stored at -70°C in sBHI containing glycerol 10% v/v.

Table I. Biotyping scheme for isolates of *H. influenzae* and *H. parainfluenzae*

Species and biotype	Production of		
	indole	urease	ornithine decarboxylase
<i>H. influenzae</i> (X + V-dependent)			
I	+	+	+
II	+	+	-
III	-	+	-
IV	-	+	+
V	+	-	+
VI	-	-	+
VII	+	-	-
VIII	-	-	-
<i>H. parainfluenzae</i> (V-dependent only)			
I	-	-	+
II	-	+	+
III	-	+	-
IV	+	+	+
V	-	-	-
VI	+	-	+
VII	+	+	-
VIII	+	-	-

Identification of haemophili

Initial species identification was based on the pattern of growth, after overnight incubation at 37°C in CO₂ 5% in air, on Blood Agar Base No. 2 (Oxoid CM271) 4% w/v around disks containing X-factor (haemin; Oxoid DD3), Y-factor (co-enzyme I; Oxoid DD4), or both (Oxoid DD5),¹⁵ combined with the results of biochemical tests performed in RapID NH System Panels for the identification of haemophili (Mercia Diagnostics, Guildford, Surrey). Subdivision into biotypes was based on tests for production of indole, urease and ornithine decarboxylase,¹ but with the modified *H. parainfluenzae* groupings suggested by Taylor *et al.*¹⁶ (table I). Isolates identified as *H. influenzae* were serotyped in a slide agglutination assay with capsular polyclonal and type b-specific antisera (Difco).

Transferrin-binding assay

This was based on the solid-phase dot enzyme assay

Fig. 1. Partial restriction map of pUO38 showing the 2.7-kb *EcoRI* fragment forming the probe for Cap b. Restriction sites shown are for *Bam*HI (B) and *Eco*RI (E). Fragment sizes shown are those generated by digestion with *Eco*RI.

described by Morton and Williams.¹¹ Strains of haemophili were first subcultured from chocolate-agar plates into sBHI containing 40 μ M FeSO₄. After overnight growth at 37°C in this iron-enriched medium, 0.1-ml volumes were subcultured into: (i) 1 ml of sBHI containing 40 μ M FeSO₄; and (ii) 1 ml of sBHI containing 25 μ M ethylenediamine di-*o*-hydroxyphenylacetic acid (EDDA; Sigma) freed previously from contaminating iron as described by Rogers.¹⁷ After overnight incubation, the cells were harvested by centrifugation in microfuge tubes, resuspended in 10 mM Tris-HCl, pH 7.4, to an OD₆₀₀ of 1.0, and 5- μ l spots were applied to a nitrocellulose membrane (0.45 μ m pore size; Sartorius, Epsom, Surrey). After drying at room temperature for 10 min, the membrane was blocked for 1 h at room temperature with skimmed milk powder 1% w/v in TBS buffer (150 mM NaCl, 10 mM Tris, pH 7.4), washed twice for 2 min each in 100 ml of TBS, and then incubated at room temperature for 2 h with a human transferrin-horseradish peroxidase conjugate (Stratech Scientific, Luton, Bedfordshire) at a final concentration of 100 ng/ml in TBS. After four washes (each for 2 min) in 100 ml of TBS, the membrane was developed with a solution consisting of 90 ml of Tris-HCl, pH 7.4, 10 ml of methanol containing 0.1 g of 4-chloro-1-naphthol (Sigma), and 100 μ l of H₂O₂. Transferrin-binding was indicated by the development of a blue dot at the site of application of the cells, and was recorded as either "constitutive" (binding after growth in the presence or absence of EDDA), "inducible" (binding only after growth in the presence of EDDA), or "no binding".

DNA hybridisation test for type b capsular genes

Preparation of Cap b DNA probe. Plasmid pUO38 (kindly provided by Dr J. S. Kroll, Department of Paediatrics, John Radcliffe Hospital, Oxford) consists of the vector plasmid pBR322 into which has been cloned a *Bam*HI fragment of c. 18 kb containing part of the Cap b region of *H. influenzae* strain Eagan.¹⁸ Fig. 1 shows a partial restriction map of pUO38. Digestion with *Eco*RI yields four visible bands after agarose gel electrophoresis: (i) 9.0 kb; (ii) two co-migrating fragments, one of which is mostly pBR322 vector DNA, of c. 4.15 kb; (iii) 2.7 kb; and (iv) 2.3 kb. Of these, the 2.7-kb fragment has been shown to form a specific probe for serotype b strains.¹⁸ This fragment was prepared, purified, and labelled with biotin-14-dATP (Gibco BRL) as described previously.¹⁹

Hybridisation experiments. Total cellular DNA was isolated from strains of haemophili by the method of Moxon *et al.*,²⁰ each microfuge tube preparation of DNA was dissolved finally in 100 μ l of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA was then applied (5- μ l spots) to a nitrocellulose membrane, air-dried, and baked at 80°C for 2 h to bind the DNA to the membrane. Before hybridisation, membranes were re-hydrated and treated with proteinase K (Sigma)

as described by Carter *et al.*¹⁹ Pre-hybridisation and hybridisation with the labelled probe was also as described previously.¹⁹ Detection of a positive hybridisation result was by means of a BlueGENE Kit (Gibco BRL) with the conditions and protocols recommended by the manufacturer.

Ability of transferrin to restore growth in iron-restricted liquid media

This was based on the method described by Holland *et al.*,²¹ with the additional step that the internal iron pools of the haemophili tested were first depleted by overnight growth at 37°C in sBHI containing 25 μ M EDDA. These "iron-stressed" cultures were then used to inoculate 250-ml flasks containing: (i) 25 ml of sBHI plus 40 μ M FeSO₄; (ii) 25 ml of BHI plus 100 μ M EDDA (iron-restricted conditions); and (iii) 25 ml of sBHI plus 100 μ M EDDA plus 90% iron-saturated human transferrin (Sigma) at 200 μ g/ml. The flasks were incubated at 37°C on an orbital shaker at 220 rpm. Growth was monitored at OD₆₀₀.

Rapid test for siderophore production

This was adapted for haemophili from the method described by Schwyn and Neilands.²² Siderophore assay agar for haemophili was prepared as follows: for each 100 ml of agar, 12.1 g of Chrome Azurol S Dye (Mordant blue 29; Sigma) was added to 5 ml of H₂O, followed by the addition of 1 ml of an iron solution (1 mM FeCl₃, 10 mM HCl). This solution was then added to 14.58 mg of cetrime (mixed alkyl trimethylammonium bromide; Sigma) dissolved in 4 ml of H₂O. The resulting blue solution was autoclaved. Concomitantly, a solution containing 3.3 g of dehydrated BHI, 1.5 g of Bacto Agar (Difco), 3.034 g of PIPES (Sigma), 1.2 ml of NaOH 50% w/v, and 0.1 ml of L- α -phosphatidyl-choline (Sigma) 10% w/v in 88 ml of H₂O was also autoclaved. The two solutions were cooled to 50°C, mixed to produce a green agar solution, and then supplemented with PPIX and NAD, as described earlier, immediately before pouring agar plates. Once the agar had set, it was inoculated with 5- μ l spots of cultures grown overnight at 37°C in sBHI containing 25 μ M EDDA. Incubation was at 37°C for up to 48 h. The presence of a yellow zone around an inoculum spot indicated the production of a high affinity iron chelator, i.e., a siderophore.

Results

Identification and transferrin-binding ability of haemophili

All of 78 invasive isolates were identified as *H. influenzae* type b. A corresponding total of 78 commensal isolates was obtained after screening cultures

Table II. Transferrin-binding ability of invasive and commensal isolates of *Haemophilus* spp.

Species	Total number of strains	Number of strains with transferrin-binding ability		
		Constitutive	Inducible	No binding
Invasive isolates (78)				
<i>H. influenzae</i> type b				
Biotype I	78	57	14	7
Commensal isolates (78)				
<i>H. influenzae</i> type b				
Biotype I	2	0	0	2
<i>H. influenzae</i> non-typable				
Biotype I	21	1	6	14
Biotype II	4	1	1	2
Biotype III	5	0	3	2
Biotype IV	1	0	1	0
Biotype V	1	0	1	0
Biotype VI	1	0	0	1
Biotype VII	1	0	0	1
<i>H. parainfluenzae</i>				
Biotype I	3	0	0	3
Biotype II	5	0	0	5
Biotype III	28	8	4	16
<i>H. segnis</i>	5	0	0	5
<i>H. parahaemolyticus</i>	1	1	0	0

from 410 routine throat swabs. Of these commensal isolates, two were *H. influenzae* type b, 34 were non-typable *H. influenzae*, 36 were *H. parainfluenzae*, five were *H. segnis*, and one was *H. parahaemolyticus*. Subdivision of these strains into biotypes is shown in table II.

Fig. 2 shows an example of the solid-phase dot

enzyme assay to illustrate the distinction between "constitutive", "inducible" and "no binding" of transferrin. Of the 78 invasive isolates, 71 (91%) were capable of binding transferrin, with 57 (73%) binding transferrin constitutively, i.e., even when grown in an iron-sufficient medium (table II). Seven invasive isolates of *H. influenzae* type b were identified that, in repeated experiments, did not appear to bind transferrin when grown in either the presence or absence of iron. Of the 78 commensal isolates, 11 (14%) strains bound transferrin constitutively. These comprised two non-typable *H. influenzae* isolates belonging to biotype I, eight *H. parainfluenzae* isolates belonging to biotype III, and one isolate of *H. parahaemolyticus*. A further 16 (21%) strains were capable of binding transferrin after growth in an iron-deficient medium. These comprised 12 non-typable *H. influenzae* isolates belonging to five different biotypes, and four *H. parainfluenzae* isolates belonging to biotype III. Two commensal isolates of *H. influenzae* type b failed to bind transferrin in either the presence or absence of iron (table II).

Analysis of isolates by DNA hybridisation with a type-b capsular probe

Direct binding of transferrin by human-associated haemophili other than *H. influenzae* type b has not been reported previously. Hybridisation with the DNA probe derived from pUO38 allows the identification of non-capsulate (b^-) progeny of type b strains.²³ All 98 strains that were capable of binding transferrin, plus the nine strains identified as *H.*

Fig. 2. Example of the solid-phase dot enzyme assay illustrating the distinction between "constitutive" (strains A and B), "inducible" (strains C and D) and "no binding" (strains E and F) of transferrin. Cultures were grown in the presence (+ Fe) or absence (- Fe) of iron as described in *Materials and methods*.

Fig. 3. Example of results obtained in dot-blot hybridisation experiments with the probe specific for Cap b. The blot shown contains total genomic DNA from 31 isolates, including the 27 "transferrin-binding" strains which were not *H. influenzae* type b (lines 2-4). The top line of four isolates comprises (left to right): *H. influenzae* type b strain Eagan; *E. coli* K12 negative control; two invasive isolates of *H. influenzae* type b from CSF.

Fig. 4. Examples of growth curves obtained with representative strains of haemophili grown in (□) sBHI + 40 μ M FeSO₄; (●) sBHI + 100 μ M EDDA; (■) sBHI + 100 μ M EDDA + 90% iron-saturated human transferrin 200 μ g/ml. A, *H. influenzae* type b ("inducible"); B, *H. influenzae* type b ("no binding"); C, non-typable *H. influenzae* ("constitutive"); D, non-typable *H. influenzae* ("no binding"); E, *H. parainfluenzae* ("constitutive").

influenzae type b that did not bind transferrin, were tested in dot-blot hybridisation experiments with the probe derived from pUO38 that was specific for Cap b. All 80 strains identified as *H. influenzae* type b, including the nine strains that failed to bind transferrin, generated a positive hybridisation signal with the probe. The remaining 27 "transferrin-binding" strains failed to hybridise with the probe (fig. 3).

Restoration of growth by transferrin in iron-restricted broth cultures

Representative strains from each group of isolates were tested for their ability to obtain iron from transferrin when grown in iron-restricted broth cultures. Examples of the results obtained are shown in fig. 4. All of the tested strains that were capable of binding transferrin were also capable of obtaining the necessary iron from transferrin for growth in broth cultures (e.g. fig. 4A,C,E). Unexpectedly, all nine of the *H. influenzae* type b strains that did not appear to bind transferrin were still capable of obtaining iron from transferrin (e.g., fig. 4B), but the non-typable *H. influenzae* strains that did not bind transferrin were unable to obtain iron from transferrin in liquid medium (e.g., fig. 4D).

Siderophore production

Apart from a direct binding mechanism, an alternative method of obtaining iron from transferrin might involve the production of diffusible siderophores. Using the rapid agar test for siderophore production, none of the *H. influenzae* type b isolates was identified as a producer of siderophores, but 18 (53%) of the non-typable *H. influenzae* isolates generated yellow zones around the inoculum spot, indicating the presence of a diffusible iron chelator. Similarly, amongst the 36 *H. parainfluenzae* isolates, 27 (75%) gave positive results, as did all five of the *H. segnis* isolates. With the exception of the nine *H. influenzae* type b isolates referred to above, strains that were non-transferrin-binding and that did not appear to produce siderophores were unable to obtain iron from transferrin in iron-restricted broth cultures.

Discussion

Haemophili are known to express various iron-uptake mechanisms, including the siderophore-dependent iron-sequestering systems possessed by *H. parainfluenzae* and *H. paraphrophilus*,⁶ and the direct transferrin receptor-mediated iron acquisition system possessed by *H. influenzae*.^{11,24} In the present study it was confirmed that the majority of *H. influenzae* type b isolates from invasive infections, as well as a smaller

proportion of non-typable commensal isolates, were capable of binding human transferrin and using it as a source of iron. Furthermore, transferrin-binding ability was also identified, for the first time, in isolates of *H. parainfluenzae* and *H. parahaemolyticus*. Although previous studies with a limited number of isolates have failed to identify this property in *H. parainfluenzae*,^{6,25} it is worth noting that transferrin-binding ability in *H. parainfluenzae* was confined in the present study to isolates belonging to biotype III. This biotype occurs relatively infrequently in the oropharyngeal flora (16% of *H. parainfluenzae* isolates from healthy controls in a recent study¹⁶), and, therefore, may not have been included in the previous surveys. However, the occurrence of transferrin-binding ability in human-associated members of the genus other than *H. influenzae* is not surprising since haemophili of animal origin also have been reported to bind transferrins derived from their respective hosts.²⁶⁻²⁹ Therefore, transferrin-binding ability may be a common property of species belonging to this genus, but one which is not necessarily shared by all individual isolates.

A previous study¹³ of fresh clinical isolates from the blood or CSF of 14 patients with meningitis found that transferrin binding was constitutive rather than iron-regulated. It has been suggested that the selective pressure of growth in the iron-restricted environment of blood or CSF might lead to a regulatory switch that results in constitutive expression of the transferrin receptor. In the present study, 91% of the invasive *H. influenzae* type b isolates from 78 patients were capable of binding transferrin, with 73% binding transferrin constitutively. Only two commensal strains of *H. influenzae* type b were isolated (both of which failed to bind transferrin), but constitutive transferrin-binding ability was also identified in two non-typable isolates of *H. influenzae*, eight *H. parainfluenzae* isolates, and one *H. parahaemolyticus* isolate. Therefore, constitutive transferrin-binding ability is associated strongly with, but is not confined solely to, haemophili isolated from invasive disease.

Of particular interest were the seven invasive and two commensal isolates of *H. influenzae* type b that did not appear to bind transferrin after growth in either iron-sufficient or iron-deficient conditions. These strains were confirmed as type b strains by hybridisation with the Cap b-specific DNA probe. There was no evidence that any of these strains produced siderophores, yet they were still able to multiply at normal rates when grown with transferrin in iron-restricted liquid medium. In this context, it is worth noting that six "non-binding" mutant derivatives, constructed in a separate study by an insertional mutagenesis procedure from a "constitutive" strain, were still able to obtain iron from transferrin when grown in iron-restricted liquid medium (K. J. Towner, unpublished results). Further studies are required to determine whether these "non-binding" strains remain capable of binding transferrin at low, undetectable levels,

or whether some haemophili have the capacity to obtain iron from transferrin by an as yet undescribed mechanism.

The present study was concerned solely with analysing transferrin-binding ability rather than studying the nature of the transferrin receptor(s) in the different strains. The transferrin receptor in *H. influenzae* is thought to consist of at least two proteins—a high M_r protein of *c.* 100 kDa (TBP1), and a lower M_r protein (TBP2) that varies from *c.* 70 to 90 kDa depending on the strain examined.^{8,11,24} Similar results have been reported for *N. meningitidis*,^{9,10,30} and the existence of shared antigenic domains in the TBPs of *H. influenzae* and *N. meningitidis*³¹ has raised the possibility that a single vaccine could confer protection against the two

major causes of bacterial meningitis. Such studies of antigenic relationships amongst TBPs have so far been performed with only a very limited number of strains. Preliminary results with the strains described in the present paper suggest that the TBPs from *H. influenzae* isolates in the Nottingham area are relatively conserved (K. R. Hardie, unpublished results), but further progress in this field will depend on detailed analysis of TBPs from isolates from a wide range of geographical locations.

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References

1. Hoiseth SK. The genus *Haemophilus*. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) *The prokaryotes*. New York, Springer-Verlag. 1992: 3304–3330.
2. Turk DC. The pathogenicity of *Haemophilus influenzae*. *J Med Microbiol* 1984; **18**: 1–16.
3. Albritton WL. Infections due to *Haemophilus* species other than *H. influenzae*. *Annu Rev Microbiol* 1982; **36**: 199–216.
4. Otto BR, Verweij-van Vught AMJJ, MacLaren DM. Transferrins and heme-compounds as iron sources for pathogenic bacteria. *Crit Rev Microbiol* 1992; **18**: 217–233.
5. Griffiths E. The iron-uptake systems of pathogenic bacteria. In: Bullen JJ, Griffiths E (eds) *Iron and infection: molecular, physiological and clinical aspects*. Chichester, Wiley. 1987: 69–137.
6. Williams P, Morton DJ, Towner KJ, Stevenson P, Griffiths E. Utilization of enterobactin and other exogenous iron sources by *Haemophilus influenzae*, *H. parainfluenzae* and *H. paraphrophilus*. *J Gen Microbiol* 1990; **136**: 2343–2350.
7. Tsai J, Dyer DW, Sparling PF. Loss of transferrin receptor activity in *Neisseria meningitidis* correlates with inability to use transferrin as an iron source. *Infect Immun* 1988; **56**: 3132–3138.
8. Schryvers AB. Identification of the transferrin- and lactoferrin-binding proteins in *Haemophilus influenzae*. *J Med Microbiol* 1989; **29**: 121–130.
9. Ala'Aldeen DA, Davies HA, Wall RA, Borriello SP. The 70 kilodalton iron-regulated protein of *Neisseria meningitidis* is not the human transferrin receptor. *FEMS Microbiol Lett* 1990; **69**: 37–42.
10. Griffiths E, Stevenson P, Ray A. Antigenic and molecular heterogeneity of the transferrin-binding protein of *Neisseria meningitidis*. *FEMS Microbiol Lett* 1990; **69**: 31–36.
11. Morton DJ, Williams P. Siderophore-independent acquisition of transferrin-bound iron by *Haemophilus influenzae* type b. *J Gen Microbiol* 1990; **136**: 927–933.
12. Anderson P, Johnson RB, Smith DH. Human serum activities against *Haemophilus influenzae* type b. *J Clin Invest* 1972; **51**: 31–38.
13. Holland J, Langford PR, Towner KJ, Williams P. Evidence for in vivo expression of transferrin-binding proteins in *Haemophilus influenzae* type b. *Infect Immun* 1992; **60**: 2986–2991.
14. Cruickshank R (ed). *Medical microbiology*, 11th edn. Edinburgh, Churchill Livingstone. 1965: 748.
15. Kilian M. *Haemophilus*. In: Lennette EH, Balows A, Hausler WJ, Truant JP (ed) *Manual of clinical microbiology*, 3rd edn. Washington, D.C., American Society for Microbiology. 1980: 330–336.
16. Taylor DC, Cripps AW, Clancy RL *et al.* Biotypes of *Haemophilus parainfluenzae* from the respiratory secretions in chronic bronchitis. *J Med Microbiol* 1992; **36**: 279–282.
17. Rogers HJ. Iron-binding catechols and virulence in *Escherichia coli*. *Infect Immun* 1973; **7**: 445–456.
18. Ely S, Tippet J, Moxon ER. Identification and characterization of a serotype b-specific segment of the *Haemophilus influenzae* genome. *Infect Immun* 1989; **57**: 2926–2928.
19. Carter GI, Towner KJ, Slack RCB. Detection of TEM beta-lactamase genes by non-isotopic spot hybridization. *Eur J Clin Microbiol* 1987; **6**: 406–409.
20. Moxon ER, Deich RA, Connelly C. Cloning of chromosomal DNA from *Haemophilus influenzae*. Its use for studying the expression of type b capsule and virulence. *J Clin Invest* 1984; **73**: 298–306.
21. Holland J, Towner KJ, Williams P. Isolation and characterisation of *Haemophilus influenzae* type b mutants defective in transferrin-binding and iron assimilation. *FEMS Microbiol Lett* 1991; **77**: 283–287.
22. Schwyn B, Neilands JB. Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* 1987; **160**: 47–56.
23. Kroll JS, Ely S, Moxon ER. Capsular typing of *Haemophilus influenzae* with a DNA probe. *Mol Cell Probes* 1991; **5**: 375–379.
24. Schryvers AB. Characterization of the human transferrin and lactoferrin receptors in *Haemophilus influenzae*. *Mol Microbiol* 1988; **2**: 467–472.
25. Herrington DA, Sparling PF. *Haemophilus influenzae* can use human transferrin as a sole source for required iron. *Infect Immun* 1985; **48**: 248–251.
26. Morton DJ, Williams P. Utilization of transferrin-bound iron by *Haemophilus* species of human and porcine origins. *FEMS Microbiol Lett* 1989; **65**: 123–128.
27. Gonzalez GC, Caamano DL, Schryvers AB. Identification and characterization of a porcine-specific transferrin receptor in *Actinobacillus pleuropneumoniae*. *Mol Microbiol* 1990; **4**: 1173–1179.
28. Ogunnariwo JA, Cheng C, Ford J, Schryvers AB. Response of *Haemophilus somnus* to iron limitation: expression and identification of a bovine-specific transferrin receptor. *Microb Pathog* 1990; **9**: 397–406.
29. Ogunnariwo JA, Schryvers AB. Correlation between the ability of *Haemophilus paragallinarum* to acquire ovotransferrin-bound iron and the expression of ovotransferrin-specific receptors. *Avian Dis* 1992; **36**: 655–663.
30. Schryvers AB, Morris LJ. Identification and characterization of the transferrin receptor from *Neisseria meningitidis*. *Mol Microbiol* 1988; **2**: 281–288.
31. Stevenson P, Williams P, Griffiths E. Common antigenic domains in transferrin-binding protein 2 of *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae* type b. *Infect Immun* 1992; **60**: 2391–2396.